GENOMICS OF FEMALE FLOWERING AND SEED YIELD IN *JATROPHA CURCAS* L.

SYNOPSIS

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BY

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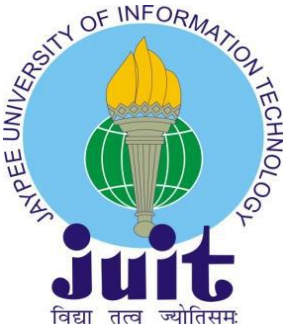

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Introduction

Today's rising demand for transport fuels along with limited sources of fossil fuels has attracted the global attention towards non-conventional source. Fuel obtained from bioenergy plants and algae has gained importance. *Jatropha curcas* L (Jatropha) has emerged as a potential bioenergy plant due to its high seed oil content (45-50%). Another favorable factor for Jatropha is that it is a non-edible crop and hence it does not affect food security. Jatropha can be grown in extreme climatic conditions, allowing the effective management of agricultural wastelands [1].

Jatropha is a monoecious perennial shrub belongs to family euphorbiaceae. The plant originated in Central America and is now well established in most tropical and sub-tropical areas of the world for its potential value as a source of biodiesel [2]. The seeds contain 45-50% oil which constitutes 21% saturated fatty acids and 79% unsaturated fatty acids (4.2% palmitic acids, 7% stearic acid, 43.1% oleic acid, 34.3% linoleic acid and 1.4% others). Due to the similarities of Jatropha oil to that of a biodiesel, it has gained importance as a potential biodiesel plant (Table 1) Major limitation in commercialization of Jatropha is its low feedstock yield (seed yield) which is governed by two major factors i.e. female to male flower ratio and count/weight of seeds per plant. The average ratio of female to male flowers in Jatropha is 1:25-30. This is resulting in only 10-15 fruits per inflorescence which is very low when compared with the number of flowers.

Table 1. Properties of Jatropha oil and diesel.

Properties	**Diesel**	**Jatropha Curcas oil**
Density(gm/cc) at 30°C	0.93292	0.836–0.850
Kinematic viscosity (cSt) at 30°C	4-8	37-52.76
Pour point (°C)	-20	-3
Cloud point (°C)	-15- -5	2
Flash point (°C)	68	210-240
Calorific value	42–46	38.20
Cetane No.	38	40–55

Jatropha is a male flower dominating plant as male flowers can develop at the female flowering site whereas at male type inflorescence's female flower number is zero [3]. It possesses a complex floral biology where female flower is present on the top whereas sub-branches may produce either female or male [4]. After floral commitment, the female flower is present as bisexual tissue and when sexual differentiation begins, abortion of male tissues occurs allowing the development of female flowers. The male flower exhibits male tissues throughout reproductive phase. There is also a middle type inflorescence where the female sites of female flowers at an inflorescence may grow as any of female flowers or male flowers or an intermediate type. These intermediates are similar to female flowers in structure but have 8-10 stamens.

The ratio of female to male flowers vary among different genotypes in Jatropha with respect to season, climate, and nutrition [4]. Genetic factors contributing to these changes in female to male flower ratio is not available. Furthermore, molecular mechanisms underlying during female flower transition is not studied yet.

Along with flowering, yield is also governed by seed numbers and seed weight. Cytokinins have proven to be the most promising growth regulator in increasing the total number of flowers and seeds. Benzyladenine and thidiazuron treatment resulted in a drastic increase in number of total flowers and female flowers along with induced bisexual flowers in Jatropha. However, fruiting rate was relatively low i.e. 2-3 folds as compared to increased female flowers with up to 9-10 folds. Also, there was a reduction in 100 seed weight in fruits formed after cytokinin treatment [5-6]. This reduction in seed weight leads to compromised seed yield even after application of phytohormones such as cytokinins. The reduction in final seed formation is also due to abortion of floral buds in cytokinin treated plants at later stages of development. This is because of all female flowers do not transit to form fruits due to higher flower abortion [7-8]. However, molecular mechanisms underlying role of central carbon flux towards biomass and overall yield, in response to exogenous phytohormone application, is not known in Jatropha. Further, no information exists on molecular signals associated with compromised seed yield following exogenous hormonal application in Jatropha. Furthermore, how exogenous application of hormone such as cytokinin affect physiological, biochemical and molecular precursors is not clear. Application of cytokinin could add carbon flux from source to sink to increase the floral biomass or seed yield. Due to these research gaps following objectives were designed:

Objective 1

Dissecting the underlying molecular mechanism during floral transitions and female flowering in *Jatropha curcas* through comparative genomics and transcript profiling.

Objective 2

To study the effect of cytokinin treatment on inflorescence meristems affecting biomass and seed yield in *Jatropha curcas.*

Objective 1

Dissecting the underlying molecular mechanism during floral transitions and female flowering in *Jatropha curcas* through comparative genomics and transcript profiling.

Materials and methods

Plant material

The shoot apical meristem with and without inflorescence and flower buds of different developmental stages were collected from Jatropha accession IC561235 planted at experimental farm of the Himalayan Forest Research Institute at Sultanpur (altitude 1400 m asl), HP, India. Plant material was frozen in liquid nitrogen immediately after collection and was stored at -80°C.

Selection of floral genes from different plant species and retrieval of their sequences

Biological information on genes involved in flowering was collected through literature survey in different plant species. All genes were classified according to their involvement in floral development, namely organ identity genes, female flowering genes, flowering transition genes, genes associated with hormonal regulation (Table 2). Nucleotide sequences were retrieved from their respective plant species. Their putative orthologous sequences were identified in Jatropha genome and annotated through FGENESH (http://www.softberry.com/berry.phtml). Specific primers were designed.

Table 2. Genes from different plant species classified according to their involvement in flower development

Category	Genes
Flowering transition genes	*ACS1, AHK2, AG, AP1, ARF2, BEL1, CEN1, CLV1, CRE1, CRY1, CRY2, CKI1, CKX1, DYAD, ETR1, FLO, FT, IPT2, IPT3, MYB98, REV, RGL, SBP, SIN1, STY, TFL1, WUS*
Floral organ identity genes	*ACS1, ACS7, AG, AP1, CUC2, DAD1, DYAD, ERAF17, ETR1, FLO, REV, SIN1, SPA, SUP, TypA1, WUS*
Female flowering genes	*ACS1, ACS7, AG, AHK1, ARF2, BEL1, CKI1, CRE1, DYAD, ERAF17, ETR1, PIN1, REV, RGL, SIN1, TypA1, WUS*
Gene associated with hormonal regulation	*AHK2, ARF2, CKI1, CKX1, CKX5, CRE1, CTR1, CYP735A, BEL1, EIN1, ERAF17, ETR1, IPT1, IPT2, IPT3, IPT9, PIN1, RGL, TAA1*

RNA isolation and cDNA synthesis

Floral buds were classified into different developmental stages after flower dissection (Fig.1). Total RNA was isolated from Jatropha tissues by using the protocol described by Sangha et al [9]. The quality of RNA was checked by 1 % (w/v) ethidium bromide-stained agarose gel. RNA was quantified in a NanoDrop spectrophotometer (Thermo Scientific) by measuring absorbance at 260nm and 280nm. First-strand cDNA synthesis was done using Verso cDNA synthesis kit (Thermo Scientific, USA) from total RNA (2 µg) template as per manufacturer's instructions.

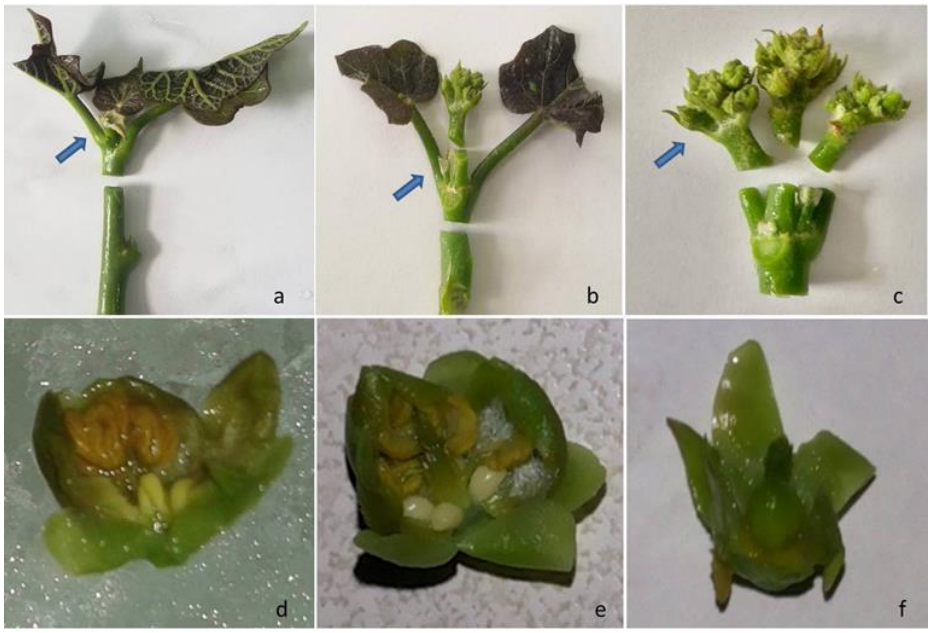

Fig. 1: Different floral developmental stages of *J. curcas*; **a.** Vegetative Stage: VS (Shoot apex without floral buds) **b.** Reproductive Stage I: RSI (Shoot apex with floral buds) **c.** Reproductive Stage II: RSII (Initial bud stage) **d.** Reproductive Stage III: RSIII (Intermediate floral bud) **e.** Reproductive Stage IV: RSIV (Male floral bud) **f.** Reproductive Stage V: RSV (Female floral bud). Arrow indicates the tissue section used for RNA extraction

Expression analysis using quantitative real time PCR (qRT-PCR)

Expression profiling of 42 genes at different floral developmental stages of Jatropha (Fig. 1) was carried out using qRT-PCR. Using gene specific primer, qRT-PCR was performed in triplicates on a CFX96 system (Bio-Rad Laboratories; Hercules, CA) with the iScript one step RT PCR kit (Bio-rad) using two biological replicates. The PCR protocol was as follows: denaturation for 3 min at 95°C, followed by 39 cycles each of denaturation for 10 s at 95°C, annealing for 30 s at 50–65°C, and elongation for 20 s at 72°C. Two housekeeping genes, 26S rRNA and GAPDH were used as internal controls for normalization.

Identification of promoter regions

Genomic sequences for genes showing differential expression and suggested to be involved in female flowering and floral transition were extracted from Jatropha genome. The gene structure was elucidated by using FGENESH. For each gene, 2 kb upstream region (potential promoter region) of the translational start site (TSS) was selected from Jatropha genome database. TSSP was used for *in-silico* identification of promoter regions. *cis* regulatory elements were identified using PLACE database (http://www.dna.affrc.go.jp/PLACE/). All cis-regulatory elements or motifs were checked for their biological functionality and important elements were identified to see commonality or differences in the regulatory control of genes with differential expression patterns.

Results and Discussion

Expression analysis at VS and RSI stages

At initial stage of flowering, shoot apical meristem receives molecular/environmental signals which transits it into inflorescence meristem (reproductive phase). Gene transcripts of *TFL1*, *SUP* and AP1 increased up to 426 folds followed by *CRY2*, *CUC2*, *TAA1* and *PIN1* with up to 5 folds at RSI as compared to vegetative phase (Fig. 2). This showed that majority of genes involved in reproductive phase transition are floral homeotic genes. Genes *TFL1*, *SUP* and *AP1* are transcription factors belonging to different classes such as TCP family, C2H2 type zinc finger protein and MADS-box family which showed higher expression in reproductive stage indicating that transcription factors might be playing major role in this transition [10-12].

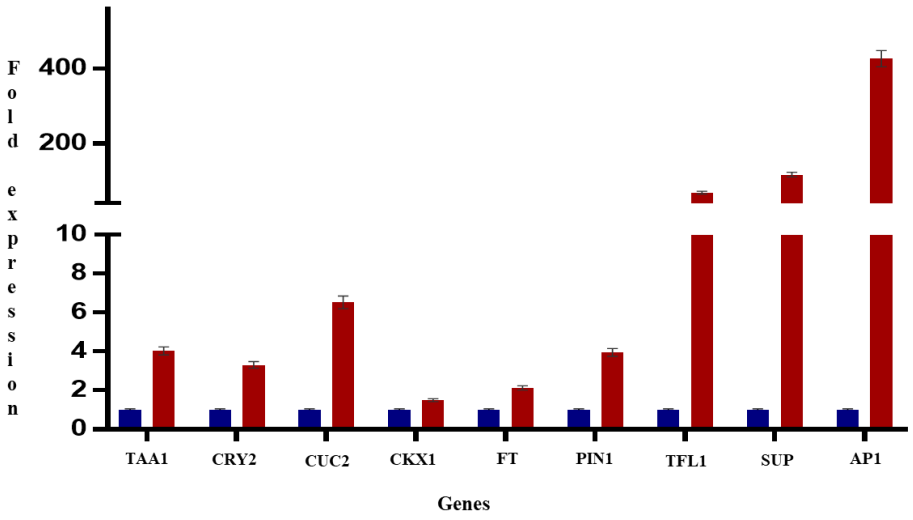

Fig. 2: Relative expression of genes associated with hormone signaling (**VS:** Vegetative Stage (Shoot apex without floral buds); **RSI:** Reproductive Stage I (Shoot apex with floral buds)

Expression analysis at RSI and RSII stages

After transition towards reproductive phase, initial bud protrudes which forms reproductive organs. Expression of *TypA1* and *AP1* increased up to ~1953 folds at RSII whereas genes *CUC2*, *RGL*, *IPT2* and *EIN2* showed increase in expression up to ~13 folds. Other genes such as *CEN1*, *BEL1*, *CRY1*, *CYP735A*, *FLO*, *FT*, *REV*, *WUS*, *STY*, *AHK2*, *CKX1*, *CRE1*, *CTR1* and *ACS1*, showed relatively higher expression at RSII (up to 8 folds) as compared to RSI (Fig. 3). Genes with higher expression in RSII were involved in cytokinin, ethylene, auxin signaling, and circadian rhythm pathway and are highly active in floral organ formation. Also, transcription factors like *CUC2*, *AP1*, *RGL* and *FLO* showed higher expression in RSII, thus involved in signaling for floral organ formation.

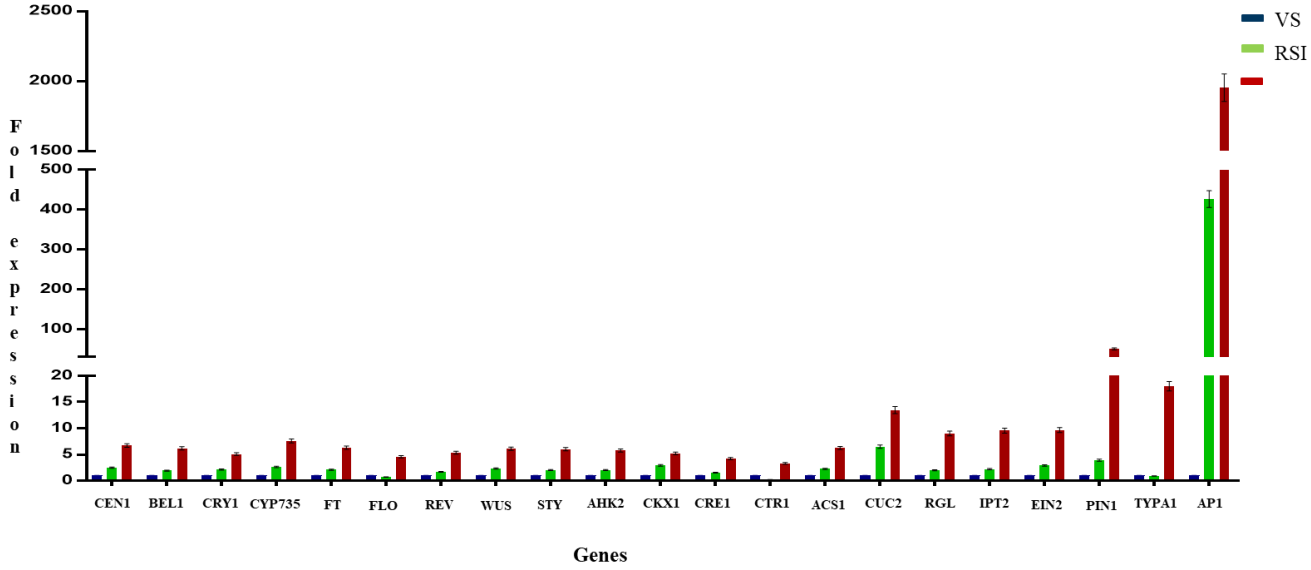

Fig. 3: Relative expression of genes associated with hormone signaling (**VS:** Vegetative Stage (Shoot apex without floral buds); **RSI:** Reproductive Stage I (Shoot apex with floral buds); **RSII:** Reproductive Stage II (Initial bud stage).

Expression analysis of genes at RSIII, RSIV and RSV stages

Initial buds further differentiate into intermediate types or male or female flower buds. Genes *CUC2*, *PIN1*, *FT*, *CKX1*, *CRY2*, *TAA1* and *SUP* possibly contribute towards female flower

formation. Genes *CKX1*, *CUC2* and *TAA1* possible contributes towards female flower development showing up to ~59 folds increase in transcript in stage RSV followed by ~18 folds, ~ 3 folds increase at RSIII and RSIV, respectively. Expression of *SUP and CRY2* gene was highest at stage RSIII with 43 folds as compared to RSIV (2 folds) and RSV (29 folds) and might be contributing towards female flower transition. *CUC2* develops female reproductive organ by controlling meristematic activity and forming boundary between meristems and organ primordia [13]. Genes *TAA1* and *PIN1* involved in auxin biosynthesis and flux are more active in RSV. These expression patterns indicated that interplay between cytokinin and auxin signaling pathways might be regulating female flower development.

Similarly, *TFL1*, *AP1* and *TypA1* genes are responsible for male flower development. *TFL1* gene showed decrease in expression from RSIII (81 folds) to RSIV (~67 folds) to RSV (~63 folds), whereas genes *AP1* and *TypA1* showed increase in expression at RSIV (1607.81 and 8.28 folds) followed by RSIII (1520.92 and 6 folds) and RSV (709 and 2 folds), respectively (Fig. 4).

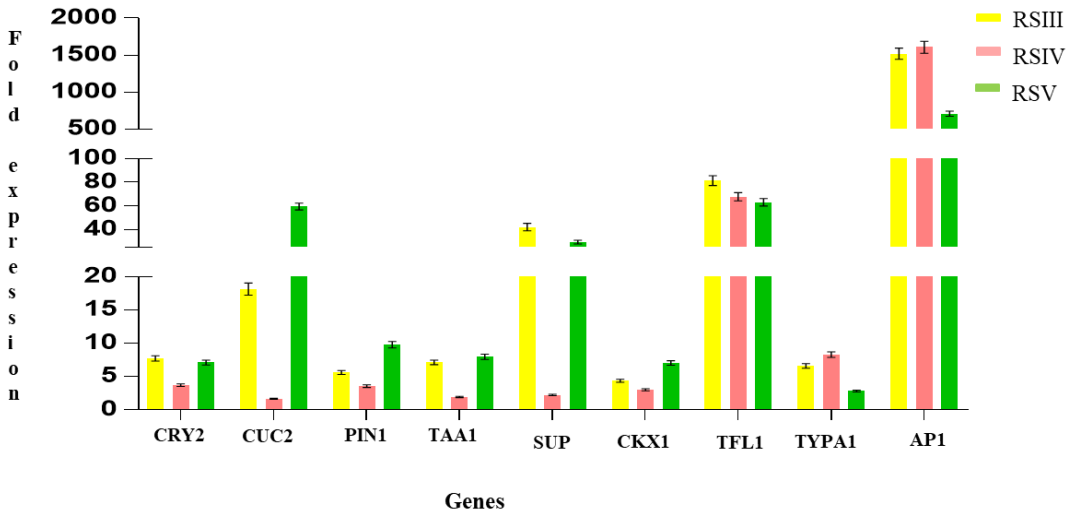

Fig. 4: Relative expression of genes associated with hormone signaling (**VS:** Vegetative Stage (Shoot apex without floral buds); **RSIII:** Reproductive Stage III (Intermediate floral bud); **RSIV:** Reproductive Stage IV (Male floral bud); **RSV:** Reproductive Stage V (Female floral bud).

Expression analysis of female flowering genes in high vs low female to male flower ratio genotypes

Genes *SUP*, *CRY2*, *PIN1*, *CKX1*, *TAA1* and *CUC2* were shortlisted through expression profiling with relatively higher expression in female flower development in a high female to male flower ratio genotype. They were further tested in RSIII, RSIV and RSV in low female flower ratio genotype. We observed when the rate of stamen abortion is high; more females are formed

indicating various internal cues are causing the arrest of stamens thus allowing female flowers to develop otherwise resulting in development of males with fused stamens. The expression of *SUP* was increased upto 7 folds in intermediate buds when compared with low genotypes and was significantly decreased in males (~0.1 folds) indicating that this might have increased the stamen abortion resulting in more female flower number [12]. Expression of *CUC2* gene showed 10-fold increase at RSV with relatively similar expression at RSIII and RSIV in high female flower ratio genotype. *TypA1* and *AP1* genes associated with male flower development showed ~2.5-fold increase at RSIII and RSIV when compared with low female flower ratio genotype (Fig. 5).

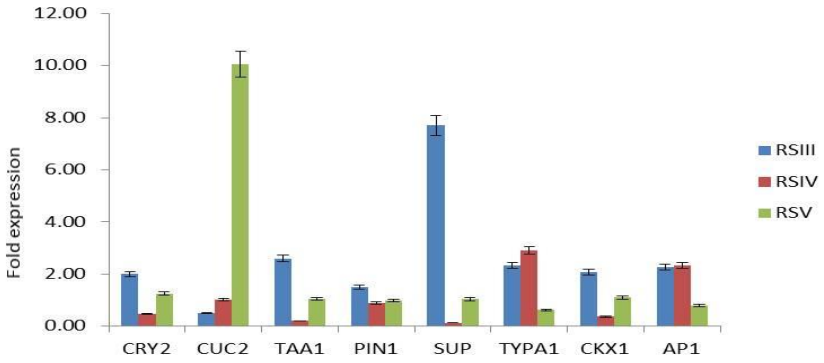

Fig. 5: Expression status of key floral genes in high (Acc. No. IC561235) versus low (Acc. No. IC569361) female to male flower ratio genotypes of Jatropha.

Correlation of gene expression to transition of female flowering

The expression of *AHK2* and *AHK4* was highest in RSII followed by RSV indicating their role in reproductive organ development and further in female flowering. Cytokinin signal transduction is mediated by receptors histidine kinases *AHK2* and *AHK4/CRE1*. *AHK2* signaling in floral development is mediated by the effector gene *CUC2* which is expressed highest in RSV [14]. *CUC2* further regulates the activity of *PIN1* which showed highest expression in RSII followed by RSV [15]. Genes *CKI1* and *PIN1* showed similar expression pattern in all the developmental stages. *CKI1* and *PIN1* follow the same expression pattern indicating correlation between endogenous cytokinin signaling and auxin flux. Therefore, *CKI1* might be regulating directly or indirectly auxin flux and thus female flowering. Gene *AHK4/CRE1*, a receptor activates *BEL1* which showed highest expression in RSII followed by RSV (Fig. 6a). *BEL1* along with *SPL* is involved in ovule development by modulating auxin fluxes through controlling *PIN1* expression [16].

SUP might be causing transition towards female flowering by blocking the expression of B class floral identity genes and aborting stamens, showed higher in RSIII followed by RSV. *SUP*,

itself is under the control of floral meristem identity gene *LFY*, which activates it through *AP3/PI*-dependent and -independent pathways [17]. Female flowering might also be controlled by circadian rhythm pathway as genes *CRY1*, *CRY2* and *FT* were expressed higher in RSV compared to RSIV (Fig. 6b). Also, *FT* and *TFL1* act antagonistically and in our study *FT* was found to be associated with female flowering while *TFL1* with the development of male flowers. Hence it is inferred that auxin and cytokinin signaling pathways and circadian rhythms control feminism in *J. curcas* and are involved in sex determination.

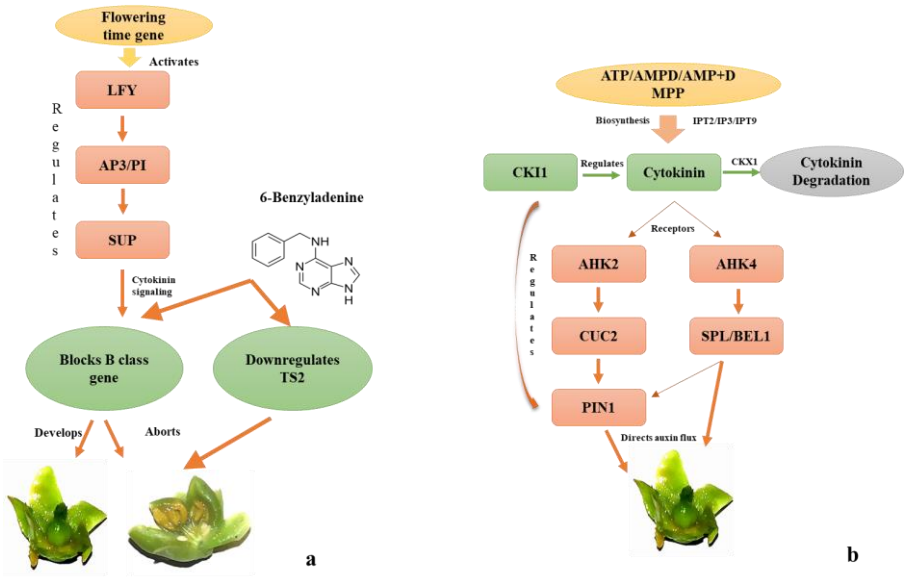

Fig. 6: Proposed model depicting possible interactions of key genes as per their expression patterns in **a.** Transition to female flowering**. b.** Development of female flowers in *Jatopha curcas***.**

In silico promoter analysis

In silico analysis of promoter regions identifies 194 motifs out of which 34 were common to all the promoters of genes showing higher expression pattern from vegetative to reproductive phase and female flowering. Promoters of genes for floral transition and development showed the presence of common elements ARR1AT, BIHD1OS, MYB1AT, POLLEN1LELAT52, and WRKY71OS. GARE2OSREP1 and CARGATCONSENSUS were present in genes involved in both VS-RSI and female flower transition in Jatropha. Sequence elements UP2ATMSD, GAREAT and MYB1AT were uniquely present in genes associated with female flowering as per expression patterns (Table 3).

Table 3. *cis* regulatory elements (common/unique) identified in the promoter regions of *J. curcas* genes with higher expression in female flowers

Regulatory Elements	IPT2	IPT3	CRY1	CRY2	CEN	BEL1	PIN1	SUP	CUC2	CRE1	AP1	TAA1
ARR1AT	+	+	+	+	+	+	+	+	+	+	+	+
BIHD1OS	+	+	+	+	+	+	-	+	+	+	+	+
MYB1AT	+	+	+	+	+	+	+	+	+	+	+	+
POLLEN1LELAT52	+	+	+	+	+	+	+	+	+	+	+	+
WRKY71OS	+	+	+	+	+	+	+	+	+	+	+	+
CARGATCONSENSUS	-	-	-	-	-	-	-	-	+	-	-	-
GARE2OSREP1	-	-	-	-	-	-	-	+	-	-	-	-
UP2ATMSD	-	-	-	-	-	+	-	-	-	-	-	-
GAREAT	-	-	-	-	-	-	+	-	-	-	-	-

Conclusion

This study provides insights into molecular basis of female flower development in a genetically superior genotype with relatively high female to male flower ratio. We conclude that genes *CUC2* and *SUP* are associated with the induction of female flowering and *SUP* arrests the development of male tissues in intermediate types in high female flower ratio genotype. These can be targeted for genetic interventions to increase the number of female flowers. Further, increase in number of female flowers could affect the seed yield, thus exogenous application hormone could be an alternative to increase the number of flowers.

Objective 2

To study the effect of cytokinin treatment on inflorescence meristems affecting biomass and seed yield in *Jatropha curcas.*

Materials and methods

Plant material and Benzyl adenine treatment

The shoot apices with and without inflorescence treated with 6-BA (160 mg/l) were collected from Jatropha accession IC561235 planted at the experimental farm of the Himalayan Forest Research Institute at Sultanpur (altitude 1400 m asl), HP, India. Shoot apical meristem tissues were frozen immediately in liquid nitrogen and stored at -80°C until use.

For cytokinin treatment, five inflorescences of five plants were sprayed with 6-BA (160mg/l). BA working solution (0.5 mM) with 0.05% (v/v) Tween-20 was sprayed onto each inflorescence meristem and the surrounding leaves using a hand sprayer. Control inflorescence meristems were sprayed with distilled water containing 0.05% (v/v) Tween-20. Spraying was done thrice at two-day interval. Shoot apices with inflorescence were collected from CONTROL (untreated), TR15 (15 days of BA treatment) and TR30 (30 days of BA treatment) (Fig. 7).

Fig. 7: *J. curcas* tissues used for transcriptome analysis. (A) Vegetative stage (VS): Shoot apex without floral buds (B) CONTROL: Shoot inflorescence meristem without BA application (C) TR15: Inflorescence meristem after 15 days of BA treatment, with increased floral buds (biomass) (D) TR30: Inflorescence meristem after 30 days of BA treatment, with less number of floral buds (biomass) as compared to TR15.

Isolation of total RNA

Total RNA was isolated from shoot apices of four stages (vegetative, control, TR15 and TR30) using RaFlex Total RNA isolation Kit (Genei) as per manufacturer's instruction. The quality of total RNA was checked on 1% denatured agarose gel (1µg) for the presence of 28S and 18S bands. The gel was run at 100 V for 30 mins. Further, total RNA was quantified using Qubit RNA BR kit (fluorometer).

Library preparation and generation of transcriptomics

The paired-end cDNA sequencing libraries were prepared using illumina TruSeq stranded Total RNA Library Preparation Kit. The raw reads were filtered using Trimmomatic (v 0.30) with quality value QV > 20 and other contaminants such as adapters were also trimmed. The Illumina NextSeq transcriptome data for three Jatropha plant samples (CONTROL, TR15 & TR30) were separately mapped to the Jatropha reference genome using BWA version 0.7.5a (http://bio-bwa.sourceforge.net/) with default parameters

Pathway analysis and differential gene expression analysis

CDSs of CONTROL, TR15 and TR30 samples were functionally annotated by KAAS (KEGG Automatic Annotation Server) with BLAST comparisons against KEGG GENES database. Expression analysis of these genes was carried out with R package DESeq and their abundance was calculated in terms of FPKM (Fragment per kilobase per million mapped reads). The FPKM values for each gene were calculated for the combination of control and treated samples with DESeq package. Gene that exhibited a p-value < 0.05 and estimated absolute log2fold (Log2fold value > 0.5 for up regulation and Log2fold value < 0 down regulation) were considered to be significantly expressed.

RT-qPCR based experimental validation

For experimental validation, RT-qPCR approach was used to confirm the observations for selected group of genes using gene specific primers in triplicate on a RT-qPCRsystem (CFX96, Bio-Rad Laboratories; Hercules CA) with the iScript RT PCR kit (Bio-rad).

Results and discussion

Pathway analysis and Differential gene expression analysis

A total of 55,755 transcripts associated with multiple pathways were expressed in CONTROL, TR15 and TR30 on the basis of FPKM values. Upon KEGG annotation, it was observed that different pathways from carbon fixation and flow to C/N ratio were upregulated in cytokinin

treatment as revealed by the genes associated to them and then downregulated after a span of 30 days (TR30). Out of these, majority of genes mapped to pathways such as photosynthesis, calvin cycle, oxidative phosphorylation, carbohydrate metabolism, nitrogen metabolism and glycolysis which implied that these were the main pathways upregulated in TR15.

Effect of cytokinin treatment on biomass and yield

BA treated inflorescence showed an increase in floral buds (biomass) upto 7-8 folds after 15 days of application and reduced to 3-4 folds after 30 days of treatment (Table 4). This might be due to the increased abortion of flowers and also reduction in photoassimilates accumulation [7, 18]. Further, yield parameters such as seed number, seed size and seed weight were recorded (after 80 days of treatment). After data analysis it is indicated the reduction in seed yield parameters like seed size and seed weight, but seed number was increased after BA application (Fig. 8; Table 5) [5-6]. These results revealed that overall yield was compromised after cytokinin application.

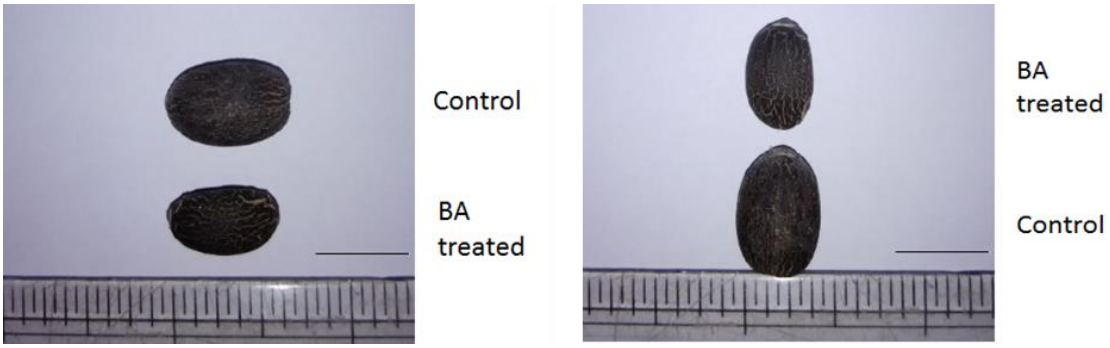

Fig. 8: Effect on seed size after cytokinin treatment

Table 4. Effect of BA treatment on floral buds at different time intervals

Parameter	Control	TR15	TR30
Number of floral buds	150	1050	500
Number of female flowers	10	-*	55-60

(*No differentiation between female and male flowers in TR15)

Table 5. Effect of BA treatment on seed yield in *J. curcas*

Yield parameter	Control	BA treated
Seed size	Length -1.8 ± 0.02cm	Length - 1.4 ± 0.03cm
	Diameter - 0.9 ± 0.012cm	Diameter - 0.7 ± 0.01cm
Seed weight	0.78 ± 0.02 g	0.48 ± 0.01 g
100 seed weight	65-67 g	50-52 g
Seed number per inflorescence	21-23	48-50

Transition from vegetative to reproductive phase

Transcriptome profiling of vegetative tissues with respect to control (shoot apices with floral buds) identified genes *FPGS1*, *BBX19*, *MED13*, *EBS*, *REV*, *MED12*, *FCA*, *MSI4*, *SPA1*, *SPA2*, *MAF5*, *FPA*, *PIN1* exclusively in the CONTROL, which denoted their association with reproductive phase transition and flowering induction. They induce flowering by repressing *FLC* [19-21]. Genes *AGL12*, *AGL17*, *AGL24*, *SPL3*, *SPL5*, *SPL15*, *CRY1* and *CRY2* showed higher transcript abundance in CONTROL as compared to VS, which inferred their involvement in transition towards flowering. They act by acts by *FT*- dependent and *FT*- independent [22-23].

Cytokinin promotes female flowering

TCP18, a stamen repressor belongs to CYC subfamily and *SUP* gene have been identified in Jatropha transcript and their increased abundance in TR15 indicated the increased stamen abortion rate [24]. Transcript level of *CKI1* and *PIN1* increased in TR15, indicating the enhanced development of female flowering. Also, *TyPA1* gene identified for male flowering has been downregulated in TR15 (Fig. 9)

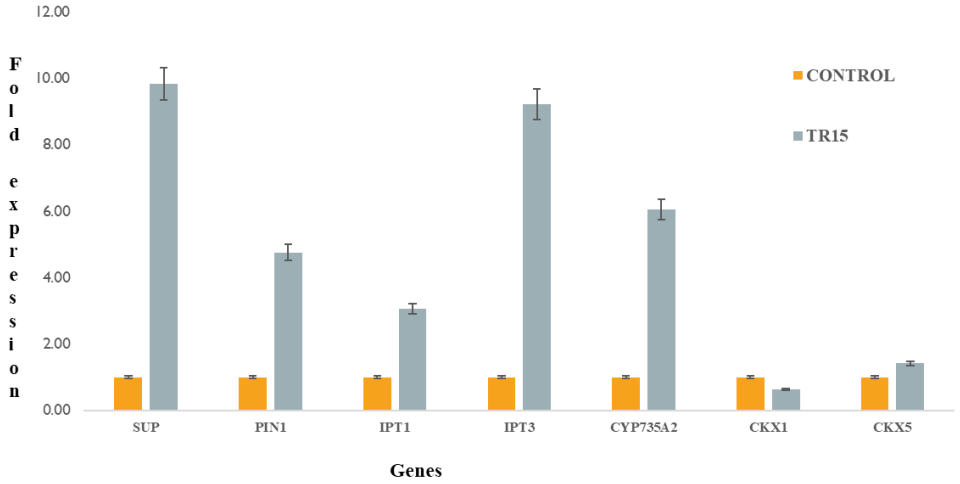

Fig. 9: Relative expression of female flowering genes in response to cytokinin application.

Effect of cytokinin application on pathways associated with carbon fixation and carbon flow

The effect of exogenous cytokinin treatment was studied at differential time intervals on pathways/genes contributing to carbon fixation and flow, which affects the overall plant (Jatropha) feedstock (Fig. 10). Differential gene expression analysis was carried out for genes associated with various pathways in three tissues (CONTROL, TR15, TR30) of Jatropha.

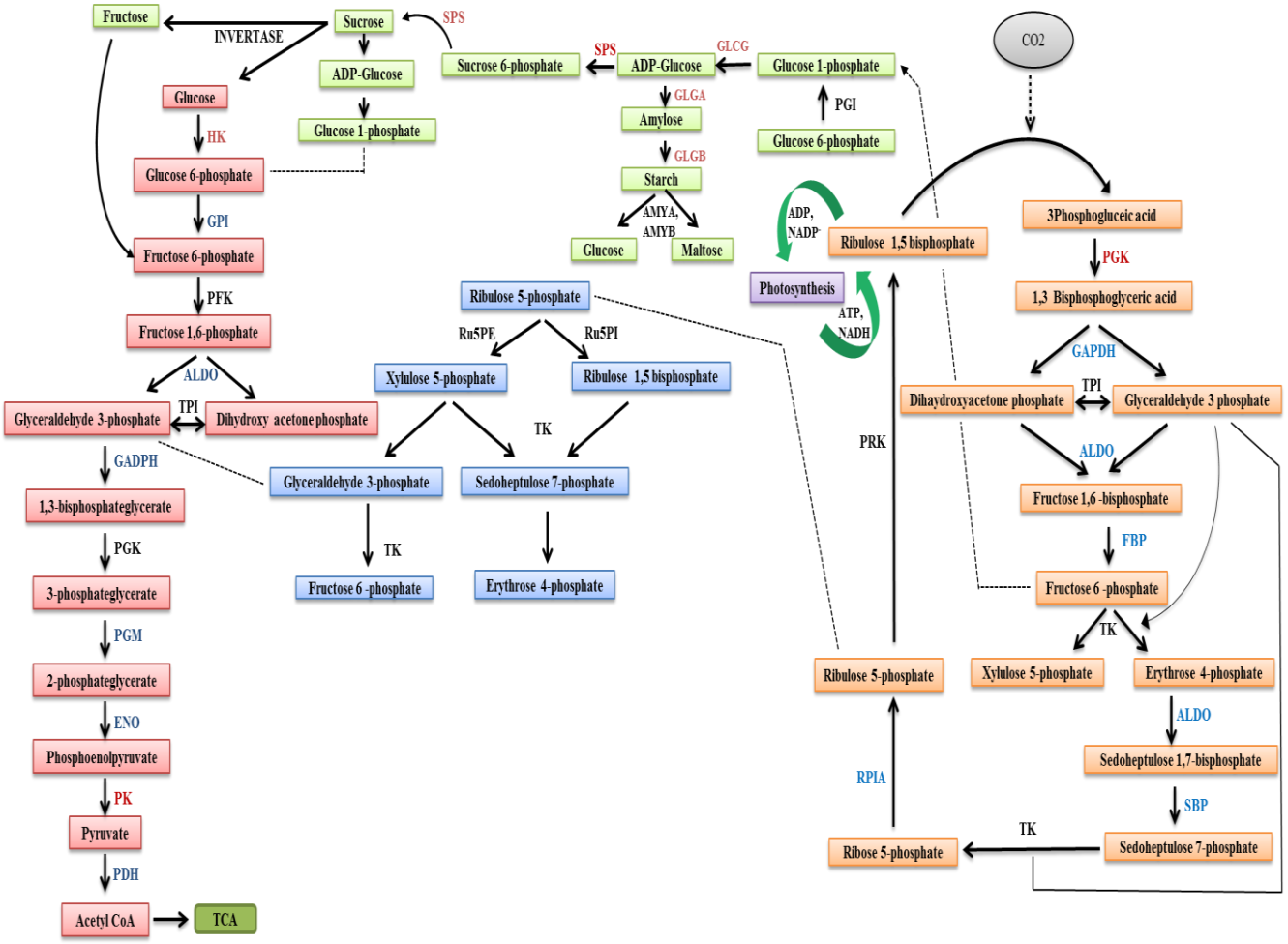

Fig. 10: Representation of enzymes upregulated and downregulated in metabolic pathways associated with carbon flux in Jatropha. Enzymes are shown in blue and red colors; Blue color signifies upregulation in TR15 and downregulation in TR30 and Red color signifies downregulation in TR30 with no significant change in TR15 and CONTROL. (RBCL- Ribulose-bisphosphate carboxylase large chain; PGK- Phosphoglycerate kinase; GAPDH- Glyceraldehyde 3-phosphate dehydrogenase; ALDO- aldolase, class I; FBP- Fructose-1,6-bisphosphatase I; TK- Transketolase; SBP- Sedoheptulose-bisphosphatase; PRK- Phosphoribulokinase; RPIA- Ribose 5-phosphate isomerase A; GPI- Glucose-6-phosphate isomerase; PGM-Phosphoglucomutase; GLGC- Glucose-1-phosphate adenylyltransferase; GLGA-Starch synthase; GLGB- 1,4-alpha-glucan branching enzyme; AMYA- Alpha-amylase;

AMYB- Beta-amylase; HK- Hexokinase; GPI- Glucose-6-phosphate isomerase; PFK- 6-phosphofructokinase 1; PDH-Pyruvate dehydrogenase).

Effect on photosynthesis

About 30 genes identified for the photosynthetic pathway, of which genes encoding *LHCI*, *LHCII*, *PSI*, *PSII* and F-type H+-transporting ATPase were upregulated in TR15 whereas showed downregulation in TR30. Genes encoding plastocyanin, ferredoxin-NADP+ reductase, F-type H+-transporting ATPase showed decreased transcript levels in TR30 compared to TR15 whereas no change was observed in TR15 as compared to control. Apart from reduction in photosynthetic pigments, the reduction in photosynthetic rate might be due to the debilitating metabolic reactions such as RuBP synthesis, ATP synthesis, and electron transfer [27]. As per our observations, the decreased transcript abundance of gene encoding enzyme NADP+ reductase in TR30 is associated with reduced photosynthetic electron flow, further limits ATPs required for carbon fixation [28]. Thus, from our findings it is imperative that the reduced biomass in TR30 is might be due to decrease in overall photosynthetic capacity.

Effect on carbon fixation

Among 18 genes identified for the calvin cycle, 12 were differentially expressed0. Genes 6-phosphogluconolactonase, fructose-1,6-bisphosphatase I (*FBP*), ribulose-bisphosphate carboxylase small chain, glyceraldehyde-3-phosphate dehydrogenase, exhibited higher transcript abundance in TR15 compared to CONTROL and TR30 whereas genes phosphoglucomutase, phosphoglycerate kinase, fructose-bisphosphate aldolase (*ALDO*), ribose 5-phosphate isomerase A, ribulose-phosphate 3-epimerase and transaldolase, sedoheptulose-bisphosphatase (*SBP*) showed higher transcript abundance in TR15 compared to TR30 but same to that of CONTROL. *FBP* and *SBP* are considered as the major control points in the Calvin cycle for CO_2 fixation and their overexpression increased the photosynthetic CO_2 fixation and carbohydrate (starch and sucrose) accumulation [29]. Thus, our observations inferred that carbon fixation was increased after 15 days but decreased after 30 days of BA treatment suggesting that reduced carbon availability might be affecting the biomass in TR30 and yield [30-31].

Effect of cytokinin application on carbohydrate metabolism

Carbon fixed through Calvin cycle is further utilized for the synthesis of carbohydrates (starch and sucrose). 23 genes were identified for starch and sucrose metabolism from which 18 genes were differentially expressed. Genes encoding enzymes linked to starch and sucrose metabolism such as glucose-1-phosphate adenylyltransferase (*AGPase*), trehalose 6-phosphate synthase/phosphatase, 4-alpha-glucanotransferase, phosphoglucomutase (*PGM*), hexokinase, 1, 4-alpha-glucan branching enzyme and sucrose-phosphate synthase were down regulated in TR30 as compared to TR15. Reduced *PGM* reduces the rate of starch accumulation [32]. *AGPase* determines the flux of carbon into starch and its reduced expression might limit the

carbon flux and starch synthesis [33]. At night, carbon stored in the form of starch is mobilized by conversion to sucrose in the cytoplasm. *SPS* plays a crucial role in carbohydrate metabolism by regulating the partitioning of carbon between starch production and carbohydrate accumulation was downregulated in TR30 [34].

Starch phosphorylase, which forms glucose 1-phosphate, which enters glycolysis for sugar breakdown. The pyruvate formed at the end of glycolysis enters the citric acid cycle (TCA) provides the precursors for carbon skeleton (Berg et al. 2002). TCA cycle plays a central role in generating ATP and providing carbon skeletons many biosynthetic processes. Overall, the citric acid cycle has been reduced after the 30 days of treatment thus reducing the carbon flow, as the enzyme ATP citrate lyase has been downregulated in TR30 [35].

Overall these observations suggest that the increased floral buds might produce more photoassimilates i.e. carbon gain and carbon assimilation, however premature exhaustion of carbohydrate reserve might trigger starvation leading to decreased biomass, further affecting yield. Thus, it is inferred that the pathways contributing towards carbon accumulation has been reduced and could not meet the increased demand due to high flowering intensity, which in turn affects the development of fruits further leading to reduction in seed weight and size, overall compromising the yield.

Table 6. Genes associated with Carbohydrate Metabolism

Sequence ID	KO ID	Enzyme name/number	Log2 fold change	
			CN vs TR15	TR15 vs TR30
Jcr4S00004_187681-189573	K01051 E3.1.1.11	pectinesterase [EC:3.1.1.11]	0.93	-1.67
Jcr4S00093_62597-69132	K00695 E2.4.1.13	sucrose synthase [EC:2.4.1.13]	0.88	-1.09
Jcr4S00103_22259-24324	K01087 otsB	trehalose 6-phosphate phosphatase [EC:3.1.3.12]	1.97	0.66
Jcr4S00282_52772-59164	K01188 E3.2.1.21	beta-glucosidase [EC:3.2.1.21]	0.67	-1.22
Jcr4S00335_29091-31621	K01193 E3.2.1.26, sacA	beta-fructofuranosidase [EC:3.2.1.26]	0.68	-1.2
Jcr4S00241_82269-83743	K01184 E3.2.1.15	polygalacturonase [EC:3.2.1.15]	1.28	-2.68
Jcr4S01628_3966-7157	K01179 E3.2.1.4	endoglucanase [EC:3.2.1.4]	0.73	-0.88

Jcr4S01990_32377-36466	K00975 glgC	glucose-1-phosphate adenylyltransferase [EC:2.7.7.27]	0.04	-0.93
Jcr4S03568_15969-20860	K16055 TPS	trehalose 6-phosphate synthase/phosphatase [EC:2.4.1.15 3.1.3.12]	0.26	-1.12
Jcr4S03968_5690-9225	K15920 XYL4	beta-D-xylosidase 4 [EC:3.2.1.37]	0.62	-1
Jcr4S04515_637-3529	K00705 malQ	4-alpha-glucanotransferase [EC:2.4.1.25]	0.46	-0.7
Jcr4S00476_22059-25615	K13648 GAUT	alpha-1,4-galacturonosyltransferase [EC:2.4.1.43]	0.79	-0.98
Jcr4S00594_60398-65518	K00696 E2.4.1.14	sucrose-phosphate synthase [EC:2.4.1.14]	-0.1	-1.42
Jcr4S00445_53788-60856	K01835 pgm	phosphoglucomutase [EC:5.4.2.2]	-0.11	-0.48
Jcr4S00679_7418-10356	K00844 HK	hexokinase [EC:2.7.1.1]	0.11	-0.47
Jcr4S01025_2525-6609	K00975 glgC	glucose-1-phosphate adenylyltransferase [EC:2.7.7.27]	0.28	-0.53
Jcr4S01214_7963-15059	K00700 glgB	1,4-alpha-glucan branching enzyme [EC:2.4.1.18]	0.12	-0.7
Jcr4S01990_32377-36466	K00975 glgC	glucose-1-phosphate adenylyltransferase [EC:2.7.7.27]	0.04	-0.93
Jcr4S00594_60398-65518	K00696 E2.4.1.14	sucrose-phosphate synthase [EC:2.4.1.14]	0.01	-1.42

Effect on nitrogen assimilation and C/N ratio

9 genes associated with nitrogen metabolism were identified, of which 6 were differentially expressed in TR15. Transcript abundance of genes glutamine synthetase, carbonic anhydrase, formamidase, nitrate reductase and nitatre/nitrite transporters was repressed in TR30 showing less rate of nitrogen metabolism. Decreased transcripts glutamine synthetase (*GS*) in TR30 decreased nitrogen availability, as it is a key enzyme assimilating inorganic nitrogen. Glutamate dehydrogenase (*GDH*) acts as a shunt to glutamate synthase cycle releasing carbon from amino compounds as keto-acids and its activity reduced the carbon release under low C/N ratios [36]. *GDH* also catalyzes the amination of 2-oxoglutarate in glutamate synthesis. This indicated that due to the repressed activity of this enzyme, carbon availability has been decreased causing reduced floral biomass in TR30 and further yield.

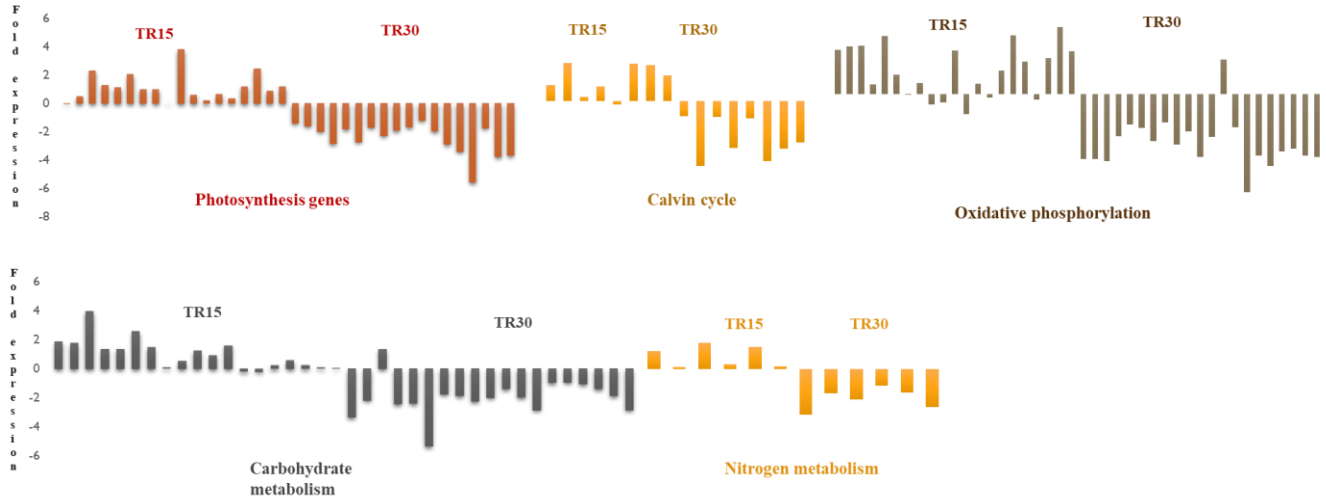

Fig. 11: Temporal representation of effect on metabolic pathways in response to cytokinin application after 15 days and 30 days of application.

RT-qPCR based experimental validation

Transcript abundance of genes from transcriptome data was validated for 31 genes and showed conjunction with *in-silico* based expression analysis (Fig. 12).

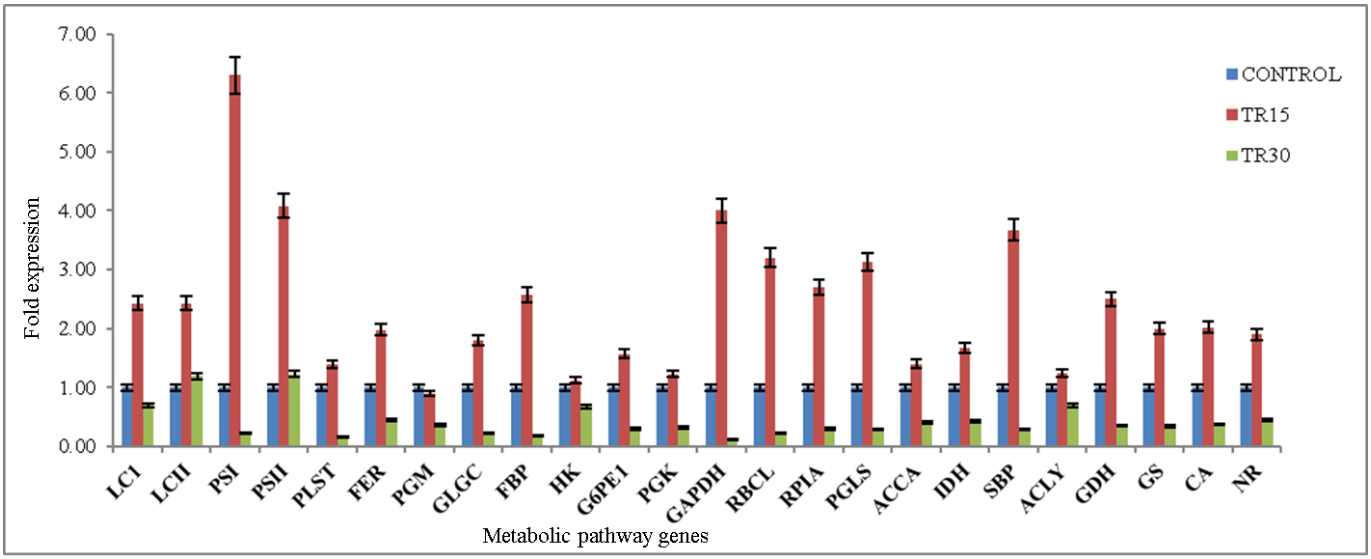

Fig. 12: RT-qPCR-based expression pattern of genes involved in photosynthesis, carbon fixation, starch and nitrogen metabolism and glycolysis. (*LCI*- light-harvesting complex I; *LCII*- light-harvesting complex I; *PSI*- photosystem I; *PSII*- photosystem II; *PLST*- plastocyanin; *FER*- ferredoxin ; *PGM*- phosphoglucomutase; *GLGC*- glucose-1-phosphate adenylyltransferase; *FBP*- fructose-1,6-bisphosphatase; *G6PE1*- glucose-6-phosphate 1-epimerase; *HK*- hexokinase; *PGK*- phosphoglycerate kinase; *GAPDH*- Glyceraldehyde 3-phosphate dehydrogenase; *RBCL*- Ribulose-bisphosphate carboxylase small chain; *RPIA*- Ribose 5-phosphate isomerase A; *PGLS*- 6-phosphogluconolactonase; *ACCA*-acetyl-CoA carboxylase carboxyl transferase subunit alpha; *IDH*-

isocitrate dehydrogenase; *SBP*- sedoheptulose-bisphosphatase; *ACLY*- ATP citrate (pro-S)-lyase; *GDH*- glutamate dehydrogenase ; *GS*- glutamine synthetase; *CA*-carbonic anhydrase; *NR*- nitrate reductase).

Conclusion

Comparative transcriptomic analysis of control and cytokinin treated shoot apical meristems at different time intervals shed light on molecular mechanisms affecting biomass and yield in Jatropha. The present study will contribute not only to genetic engineering and breeding of Jatropha for better yield but also provided insights into the mechanisms associated with effect of cytokinin treatment on carbon fixation, carbon availability and nitrogen metabolism which further affects biomass production and thus feedstock yield of Jatropha. Through this study, genes associated with carbon flux mechanisms, have been emerged as possible targets to increase the total biomass and yield in Jatropha.

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List of Publications

PUBLICATIONS

1. **M. Gangwar**, H. Sood and R.S. Chauhan. Multiple genes associate with transition to flowering and female flower development in a high female to male flower ratio genotype of *Jatropha curcas* L. Molecular Biology Reports 2016; 43(4): 305-22. **(Scopus), UGC Journal list No. 31535 [IF-1.82]**

2. **M. Gangwar**, A. Sood, A. Bansal and R.S. Chauhan. Comparative transcriptomics reveals a reduction in carbon capture and flux between source and sink in cytokinin treated inflorescences of J*atropha curcas* L. 3 Biotech 2018; 8:64. **(Scopus), UGC Journal list No. 99 [IF-1.36]**

3. **M. Gangwar**, S. Sharma, H. Sood H and R.S. Chauhan. Indirect shoot organogenesis in Jatropha curcas for in vitro propogation. Indian Journal of Research 2015; 4(12): 56-8.

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1. **M. Gangwar** and R.S. Chauhan. Dissecting the molecular basis of female flower in *Jatropha curcas*. Proceedings of the National Symposium on Advances in Biotechnology for Crop Improvement [Eternal University, Badu Sahib, HP: July 12, 2014]

2. R.S. Chauhan, A. Sood and **M. Gangwar**. Genomics-Assisted Genetic Improvement of *Jatropha curcas*. Jatropha Updates 2014. [TERI, New Delhi: October 13, 2014]